RESEARCH LETTER

The *galU* gene expression in *Streptococcus pneumoniae*

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Abstract

The polysaccharide capsule of *Streptococcus pneumoniae* is the main virulence factor making the bacterium resistant to phagocytosis. The *galU* gene of *S. pneumoniae* encodes a UDP-glucose pyrophosphorylase absolutely required for capsule biosynthesis. *In silico* analyses indicated that the *galU* gene is co-transcribed with the *gpdA* gene, and four putative promoter regions located upstream of *gpdA* were predicted. One of them behaved as a functional promoter in a promoter reporter system. It is conceivable that the sequence responsible for initiating transcription of *gpdA-galU* operon is an extended −10 site TATGATA(T/G)AAT. Semi-quantitative real-time reverse transcription PCR experiments indicated that *galU* was expressed mainly in the exponential phase of growth.

Introduction

Streptococcus pneumoniae is a leading human pathogen causing both mucosal (such as otitis media and pneumonia) and systemic diseases (including septicemia and meningitis). To date, 93 different pneumococcal capsular types have been described (Henrichsen, 1995; Park *et al.*, 2007; Bratcher *et al.*, 2010; Calix & Nahm, 2010). This remarkable phenotypic variability appears to be present also at the genetic level (Bentley *et al.*, 2006). Early studies showed that uridine diphosphoglucose (UDP-Glc) is a key component in the biosynthetic pathway of pneumococcal capsular polysaccharides containing glucose, galactose, and/or UDP-glucuronic or UDP-galacturonic acids (Mills & Smith, 1965). At least one of these sugars is a component of every capsular polysaccharide of *S. pneumoniae* (Kamerling, 2000). The enzyme UTP-Glc-1-phosphate uridylyltransferase (UDP-Glc pyrophosphorylase; EC 2.7.7.9) is encoded by the *galU* gene. This enzyme catalyzes the formation of UDP-Glc, which is the substrate for the synthesis of UDP-glucuronic acid. Also, UDP-Glc is also required for the interconversion of galactose and glucose by way of the Leloir pathway (Frey, 1996).

Previously, the *galU* gene was cloned and overexpressed, and the gene product was biochemically characterized (Mollerach *et al.*, 1998; Bonofiglio *et al.*, 2005). In addition, knockout *galU* mutants of type 1 and type 3 pneumococci are unable to synthesize a detectable capsular polysaccharide. Southern blot hybridization experiments using DNAs prepared from pneumococcal isolates belonging to different types showed that every strain tested contained a *galU* homologue (Mollerach *et al.*, 1998). Thus, the UDP-Glc pyrophosphorylase, which is directly involved in the synthesis of the capsular polysaccharide in *S. pneumoniae*, might represent a suitable target in the search for inhibitors to control the biosynthesis of the main pneumococcal virulence factor. In this sense, it should be emphasized that eukaryotic UDP-Glc pyrophosphorylases appear to be completely unrelated to their bacterial counterparts, suggesting the possibility that putative inhibitors of the bacterial enzymes would not be harmful for the host.

The *gpdA* gene (also named *gpsA*) is located immediately upstream of the *galU* gene and is predicted to encode a glycerol-3-phosphate dehydrogenase [NAD(P)(+)], (EC 1.1.1.94). The *spr1901* gene, which is annotated as a possible transcriptional regulator, is located upstream

of the *gpdA* gene and transcribed from the opposite strand. Nothing is known about the promoter region of *galU* and its differential expression at different growth phases. In this report, we identified a promoter-active DNA sequence from *S. pneumoniae* located upstream of *gpdA* that is involved in controlling the expression of *galU* through co-transcription with *gpdA*. These findings provide insight into the expression of GalU, an enzyme with a key role in virulence.

Materials and methods

Bacterial strains and growth conditions

Streptococcus pneumoniae 406 and M31 were grown in liquid C medium (Lacks & Hotchkiss, 1960) supplemented with 0.08% of each yeast extract (CY medium) and bovine serum albumin without shaking, or on reconstituted tryptose blood agar base plates (Difco Laboratories Inc., Detroit, MI) supplemented with 5% defibrinated sheep blood. Lincomycin (0.6 μg mL^{-1}) was added when required. *Streptococcus pneumoniae* M31 (Δ*lytA*) is a non-encapsulated, serotype 2 ($S2^-$) mutant having a deletion of at least 5.5 kb containing the gene *lytA* that encodes the main pneumococcal autolysin (Sánchez-Puelles *et al.*, 1986). *Streptococcus pneumoniae* 406 is a clinical isolate of serotype 3 (García *et al.*, 1993).

Escherchia coli C600 cells (*thi*-1, *leuB*, *thr*-1) were grown in Luria–Bertani growth medium (LB) at 37 °C or on LB solid agar supplemented when necessary with tetracycline (20 μg mL^{-1}; Sambrook *et al.*, 1989).

DNA techniques and plasmid construction

Restriction enzymes, T4 DNA ligase and the Klenow fragment of DNA polymerase were obtained commercially and used according to the recommendations of the suppliers. Chromosomal DNA from *S. pneumoniae* 406 was prepared as previously described (Fenoll *et al.*, 1994; Wilson, 1997). PCR was performed using standard conditions with AmpliTaq DNA polymerase (PerkinElmer). The primers used are listed in Table 1. An SphI/BbuI restriction site was included in oligonucleotides pGalU1, pGalU3, pGalU5, and pGalU7 and an SmaI restriction site in pGalU2, pGalU4, pGalU6, and pGalU8. Four different DNA fragments (F1–F4), one overhanging the other and containing putative promoter regions, were amplified by PCR.

Plasmid pLSE4 is a promoter probe vector able to replicate in *S. pneumoniae* and *E. coli* that contains a promoterless *lyt*A gene (Díaz & García, 1990). DNA fragments were ligated separately on pLSE4 previously digested with XbaI, filled with Klenow fragment and digested with BbuI.

Transformation procedures

Escherichia coli C600 was made competent and transformed with derivatives of pLSE4 as described elsewhere (Muñoz *et al.*, 1997). The accuracy of the constructs was confirmed by nucleotide sequencing of the corresponding insert. Plasmid derivatives of pLSE4 containing DNA fragments (F1–F4) were transformed into *S. pneumoniae* M31.

Streptococcus pneumoniae was transformed with plasmid DNA by treating cells in C medium supplemented with 0.08% of bovine serum albumin with synthetic competence-stimulating pheromone (250 ng mL^{-1}) at 37 °C for 10 min to induce competence (Moscoso & Claverys, 2004) followed by incubation at 30 °C during DNA uptake. *Streptococcus pneumoniae* clones obtained upon transformation with derivatives of pLSE4 were scored on CY agar plates containing lincomycin (0.6 μg mL^{-1}) and catalase (250 units mL^{-1}).

Assay for lytic activity

Crude sonicated extracts were obtained as previously described from mid-exponentially growing cultures for *S. pneumoniae* M31 derivatives (Ronda *et al.*, 1987). Assays of cell wall lytic (*N*-acetylmuramoyl-L-alanine amidase; NAM-amidase) activity were performed according to standard procedures described elsewhere using [methyl-^{3}H]choline-labeled pneumococcal cell walls as substrate (Höltje & Tomasz, 1976). One unit of NAM-amidase activity was defined as the amount of enzyme needed to catalyze the hydrolysis (solubilization) of 1 μg of cell wall material in 10 min at 37 °C.

RNA isolation, cDNA synthesis, and RT-PCR

Total RNA was extracted from *S. pneumoniae* cultures in CY medium using the RNeasy mini Kit (QIAGEN). Cells were harvested throughout the growth curve at 37 °C ($A_{550\,nm}$ of 0.12, 0.33, 0.67, and 0.66 that corresponds respectively to early, medium logarithmic, late logarithmic, and stationary growth phase) and stored in ice. Pellets were resuspended in 0.9% NaCl solution and stored at −80 °C. The concentration and the purity were estimated using an ND1000 Spectrophotometer (Nanodrop Technologies). Primers used for qRT-PCR are listed in Table 1.

cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. To ensure that the amplification observed in the PCRs was attributable to the cDNA template made from mRNA and not from contaminating genomic DNA, controls were carried out for each sample under the same conditions, except that transcriptase was not added to the reactions. Semi-quantitative real-time

Table 1. Oligonucleotide primers used in this study

Primer name	Nucleotide sequence (5′-3′)*	Purpose on use	Position†
pGalU1	AG**GCATGC**AAGTGCAAAATCCC	Cloning F1	585–606
pGalU2	TTG**CCCGGG**AAGATTTCCCCAA	Cloning F1	767–746
pGalU3	AA**GCATGC**AAAAACAAACCGTCGCCGT	Cloning F2	650–676
pGalU4	TAT**CCCGGG**TTAAGACCACTCATT	Cloning F2	1679–1656
pGalU5	CTC**GCATGC**TACAAGAACTTCCTG	Cloning F3	436–459
pGalU6	TTG**CCCGGG**CATTACTGGGGTAT	Cloning F3	600–578
pGalU7	TTAA**GCATG**CTCACCAGAAGGACAGTA	Cloning F4	–8–18
pGalU8	CAA**CCCGGG**AGGTTCTTGTAGGTTG	Cloning F4	464–440
galurtcD	CCACGCGGTCTCGGAGAT	RT-PCR	2010–2027
galurtcR	GTTGTTTGGTAAGTGGAACA	RT-PCR	2136–2117

*Bold-faced letters indicate nucleotides introduced to construct the appropriate restriction sites.
†Position is given relative to the last nucleotide of *spr1901* (see Fig. 1).

PCR (RT-PCR) experiments were performed using SYBR Green technology in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each experiment was carried out in triplicate, so each relative gene expression reported for each point of the curve represents the average of three independent biologic replicates. Changes in sample gene expression were measured based on an external standard used as a calibrator (Wong & Medrano, 2005). Dunnet's test was used to determine whether the expression values of a given point were significantly different from other points of the curve.

Genome analysis and multiple-sequence alignments

Sequences of *S. pneumoniae* genomes were retrieved from the NIH GenBank database (http://www.ncbi.nlm.nih.gov/genome?term=streptococcus%20pneumoniae). Multiple-sequence alignments were performed using the CLUSTALW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Results and discussion

In silico analysis indicates that *galU* and *gpdA* genes are co-transcribed

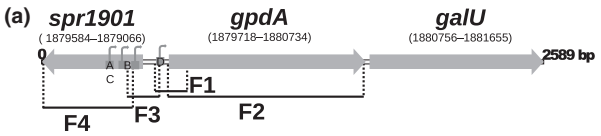
The analysis of the 23 *S. pneumoniae* genomic complete sequences available at the NIH website (http://www.ncbi.nlm.nih.gov/genome?term=streptococcus%20pneumoniae) revealed that *galU* and *gpdA* are adjacent and in the same orientation in the *S. pneumoniae* chromosome. Transcriptional terminator prediction was made using TransTermHP (http://transterm.cbcb.umd.edu/index.php). TransTermHP was run on seven complete *S. pneumoniae* genomes currently available at this site. The search process indicates that no terminator is present after *gpdA* gene although a rho-independent transcriptional terminator was found downstream of *galU*. In the case of *S. pneumoniae* R6, a predicted terminator was found with a confidence value of 70, which is regarded as high (Kingsford *et al.*, 2007).

The *gpdA* and *galU* genes are located together and are transcribed from the same DNA strand in 61 different genomes belonging to the *Firmicutes* phylum. However, the *galU* gene and its flanking regions do not have the same organization in other bacterial species not closely related to *S. pneumoniae* (Varón *et al.*, 1993; Dean & Goldberg, 2002; Silva *et al.*, 2005).

Identification of a functional *gpdA-galU* promoter region

Promoter prediction on the 827-bp sequence upstream of the *gpdA* gene was carried out using the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html). Four sequences were detected by this program as putative promoters with a score of at least 0.88 (Fig. 1).

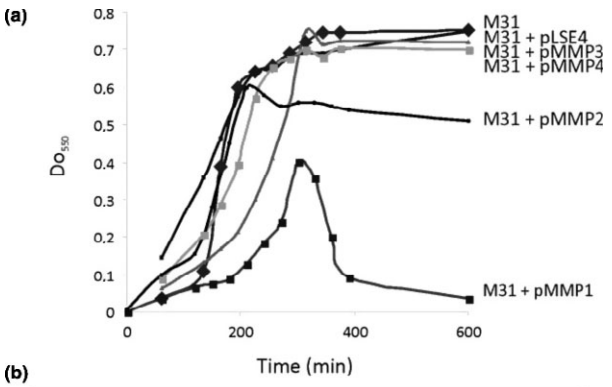
To determine whether the proposed promoter sequences actually represent a *gpdA-galU* promoter, three DNA fragments, one overhanging the other (F1, F3, and F4) and containing the putative promoters, were PCR-amplified. A 1030-bp DNA fragment (F2) containing full-length *gpdA* gene was also amplified to explore the existence of a promoter region within this gene. After digestion with the appropriate restriction enzymes, the DNA fragments were ligated to the promoter probe vector pLSE4 previously treated with the same enzymes and used to transform competent cells of *E. coli* C600. The recombinant plasmids were transferred to pneumococcal M31 strain (Δ*lytA*). Lincomycin-resistant M31 transformants, harboring different recombinant plasmids designated pMMP1 to pMMP4, were obtained. *Streptococcus pneumoniae* M31 harboring pMMP1 lysed at the end of the exponential phase of growth (Fig. 2). Moreover, a detectable LytA amidase activity (7.4 U mg^{-1} of protein) was found in sonicated extracts prepared from M31 harboring pMMP1, indicating the existence of a functional promoter in the F1 fragment.



(b) Putative promoters

A: tctccatttttcaattctaattaattgacggatacttaatt**c**agactcatc
B: gatttttttagccaaacgtaatctttttaatttttttcgcca**a**actctcgca
C: tcctgagttgtttacctctattataagcatatactgaatc**a**aactatcta
D: atgcaagtgcaaaatcccctagaatatgatagaataagag**a**aagaaatct

Fig. 1. (a) Map of the 2.589-kb region containing the *galU* gene and putative promoters predicted by NNPP software (cut off > 0.88) (A–D). Numbers in brackets indicate the nucleotide positions corresponding to the sequence included in the GenBank database under accession number AE007317.1. Fragments F1–F4 were cloned in pLSE4 reporter plasmid generating pMMP1 to pMMP4 derivatives. (b) Putative promoter sequences (A–D). Putative starting point of each sequence is bold-faced.



(b)

Strain	LytA amidase activity (U mg^{-1}protein)
M31(pLSE4)	0
M31(pMMP1)	7.43
M31(pMMP2)	0
M31(pMMP3)	0
M31(pMMP4)	0

Fig. 2. (a) Functional characterization of *gpdA-galU* promoters. (a) Growth and lysis curves of *Streptococcus pneumoniae* M31 (Δ*lyt*A) strain harboring plasmids pLSE4, pMMP1, pMMP2 pMMP3, or pMMP4. (b) Pneumococcal cell wall hydrolyzing activity of cell extracts obtained by sonication.

M31 cells containing pMMP2 also exhibited lysis at the end of exponential phase of growth although with a rate three times lower than that of the pMMP1 derivative. Moreover, LytA amidase activity was undetectable in sonicated extracts of this strain. By contrast, strains containing pMMP3, and pMMP4 and the promoterless vector (pLSE4), did not show any lysis (Fig. 2).

Sequence analysis of the region upstream *gpdA-galU*

The region located immediately upstream of *gpdA* is highly conserved in pneumococcal genomes (Fig. 3a) and was searched for the presence of promoter-like sequences. A consensus, −10 extended promoter (TATGATATAAT; Sabelnikov *et al.*, 1995) was identified in the six pneumococcal strains, and its single-nucleotide variant TAT GATAgAAT was found in the rest of the 23 genome sequences analyzed. Changes in this nucleotide are not critical for the binding of the σ subunit of the RNA polymerase (Sevostyanova *et al.*, 2007). A Shine- and Dalgarno-like sequence (SD) (AGGAGG) is located five nucleotides upstream of the *gpdA* start codon (AUG). As expected, the polycistronic transcript has a putative transcription start site within a purine (A) nucleotide located seven nucleotides downstream of the −10 extended promoter element. The putative promoter sequence consists of an extended −10 element without a −35 site and is situated 30 nucleotides upstream of the ATG initiation codon of the *gpdA* gene.

Regarding pneumococcal promoters, we compared similar extended −10 elements of *sulA* (dihydropteroate synthase), *murB* (UDP-*N*-acetylenolpyruvoylglucosamine reductase), *pcrA* (ATP-dependent DNA helicase), *comCDE* operon, and *fcsR* (regulator of fucose operon) with the *gpdA-galU* promoter described herein (Chan *et al.*, 2003; Ware *et al.*, 2005; Ruiz-Masó *et al.*, 2006 and Martin *et al.*, 2010). These sequences match the consensus extended −10 region previously described by Sabelnikov *et al.* (1995) (TnTGnTATAAT). The nucleotides in the canonical −10 hexamer TATAAT are conserved i*n murB, pcrA, comCDE, fcsR,* and *gpdA-galU* (six strains). Moreover, −10 extended promoter element of *comCDE* showed an alteration of the T-TG extension. Also, −10 and −35 promoter elements found in *fcsK* (fuculose kinase), *yefM-yoeB* (toxin–antitoxin), *relB2* (antitoxin), and *tts* (βglucosyltransferase) genes were compared, and most of them showed −10 canonical element (TATAAT) and the −35 box (TTGACA) with minor differences (Llull *et al.*, 2001; Chan *et al.*, 2003, 2011; Nieto *et al.*, 2006). On the other hand, *ung* (DNA-uracil glycosylase)

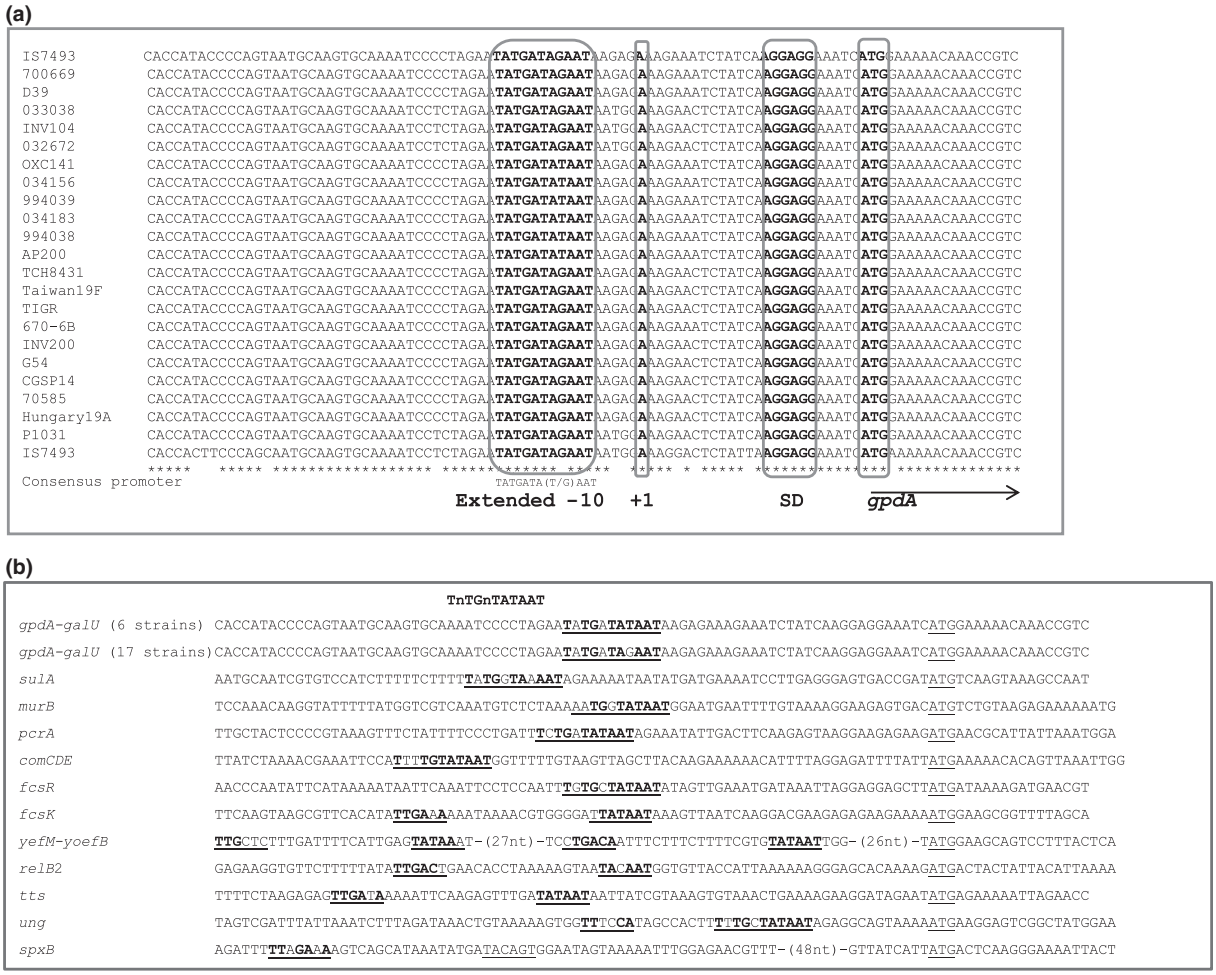


Fig. 3. (a) Multiple-sequence alignment of *gpdA-galU* promoter region from *Streptococcus pneumoniae* complete genome sequences deposited in databases. (b) Promoter regions of different pneumococcal genes are shown. *sulA, murB*, *pcrA,* comCDE, and *fcsR* genes have proven −10 extended elements; *fcsK, yefM-yoefB, relB2, tts* genes present −10 and −35 elements; *ung* and *spxB* exhibit −10 extended and −35 elements. Extended −10 sites, canonical −10 and −35 boxes are underlined. Conserved nucleotides are bold-faced. The consensus promoter sequence described by Sabelnikov is given: TnTGnTATAAT. The numbers of nucleotides that are not showed in *yefM-yoefB* and *spxB* genes are indicated in brackets. The sequences of the genes were taken from references cited in the text.

Table 2. Relative expression of *galU* gene of *Streptococcus pneumoniae* 406 during different growth stages

Growth stage	Early exponential	Mid-exponential	Late exponential	Stationary phase
Relative *galU* expression	2.4 ± 0.26	2.1 ± 0.32	2.6 ± 0.56	0.17 ± 0.06

qRT-PCR analysis was used to determine *galU* transcript abundance at different time points during growth. The transcription data are the means ± standard deviations of three independent experiments.

and *spxB* (pyruvate oxidase) exhibit −10 extended promoter element and a −35 box (Ramos-Montañez *et al.*, 2008; Ruiz-Cruz *et al.*, 2010), (Fig. 3b).

Expression of the *galU* gene is higher during exponential phase

Semi-quantitative real-time reverse transcription PCR (RT-PCR) was used to compare relative transcriptional abundance of *galU* transcripts at different points of the growth curve of *S. pneumoniae* R6. The results showed that *galU*-specific transcript levels in the exponential phase were 14-fold higher than during the other phases (Dunnet's test, $P < 0.05$; Table 2), in agreement with that previously suggested for metabolic genes involved in biosynthetic processes whose expression is probably down-regulated in the stationary phase (Navarro Llorens *et al.*, 2010). However, our results contrast with those carried out in *Bacillus subtilis* (Varón *et al.*, 1993) and *Lactobacillus*

casei (Wu *et al.*, 2009) where the expression of the corresponding *galU* genes increased in the stationary phase.

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